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14. ABSTRACT

No form of disease prevention has had greater success than prophylactic immunization. Whereas "therapeutic cancer vaccines" have only marginal evidence of clinical efficacy, prophylactic vaccination against tumor-associated antigens can confer life-long protection in both transplantable and transgenic cancer models. Yet most non-virally associated cancers lack candidate antigens that could be targeted for human cancer prevention. Endogenous Retroviral sequences and other transposable elements (TEs) comprise almost 40% of the human genome. These remnants of exogenous retroviruses are crippled through mutation and do not make infectious virus. Furthermore, promoter and histone modification silence their expression. Nevertheless, many cancers express them as a result of altered gene regulation, and the proteins they encode are recognizable as "neo-antigens" in both mouse and man. We have created "repeat sequence arrays" for the mouse and human genomes. Using mouse lung cancer models and human clinical samples, we are defining the pattern and kinetics of TE expression during neoplastic transformation. We seek to determine if endogenous immune responses to such antigens may lead to early detection, and if prophylactic vaccination delays or prevents the onset of lung cancer in mouse models.

15. SUBJECT TERMS
Transposable elements, prophylactic vaccination, repeat sequence arrays

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INTRODUCTION:

Prophylactic vaccination against infectious pathogens has been the most effective form of disease prevention in the history of medicine. The dream of applying this powerful modality to the prevention of other diseases such as lung cancer however, is hampered by a lack of knowledge of the relevant neo-antigens that cancers that have yet to arise, will subsequently express. In particular, the identity of those antigens expressed early in the course of neoplastic transformation prior to systemic dissemination is critically lacking. The central focus of this one-year pilot project was to evaluate the temporal expression of a distinct class of tumor associated antigens, i.e. the products of aberrantly expressed endogenous retroviral sequences or other transposable elements (TEs) in a mouse model of spontaneous lung cancer. The central hypothesis being tested is that such de-repressed expression will occur early in the course of transformation, and that the particular pattern of TE expression will reflect the genetic and epigenetic pathways involve in transformation, rather than being a purely stochastic event. If correct, such findings will support the exploration of establishing prophylactic immunity to such antigens prior to malignant transformation as a means to impact on the development of clinical overt cancers.

BODY:

The funded proposal embodied a Scope of Work that contained three tasks as summarized below. Progress and findings are described after each task.

Task 1- *Design and create oligonucleotide microarrays of repetitive DNA sequences present in the mouse genome.*

Traditional commercially available gene expression microarrays, a fairly cheap and quick assaying technology, mask out TEs on the premise that they are not functional and/or do not encode proteins relevant to cell function, thus preventing their use in high throughput expression studies.

In the first task, we designed a Transposable Element Array (TE-Array), a high-throughput tiling microarray that probes all annotated TEs in both sense and antisense orientation in the mouse genome.

Several QC tests were run to validate and pass TE-Array in terms of sensitivity and specificity before proceeding to experimental samples. The arrays were designed as described in the project narrative, and printed replicates were produced by Agilent Technologies. The performance of the arrays was validated using RNA isolated from several normal mouse tissues (Lung, breast, prostate, brain, testes). Normal tissues were compared between male and female mice of two different mouse strains (BALB/c and C57BL/6). For comparison, the transplantable lung cancer cell line Lewis Lung Carcinoma (LLC) was also used. Biological replicates were run using LLC to assure reproducibility of the pattern of expression. The performance of the arrays was also validated using the positive controls incorporated into the array design, which include a number of oncogenes and tumor suppressor genes whose over or under-expression in lung cancers relative to normal tissues has been well documented. Differences in the observed tissue expression patterns relative to the Lewis Lung Carcinoma are displayed in figure 1.

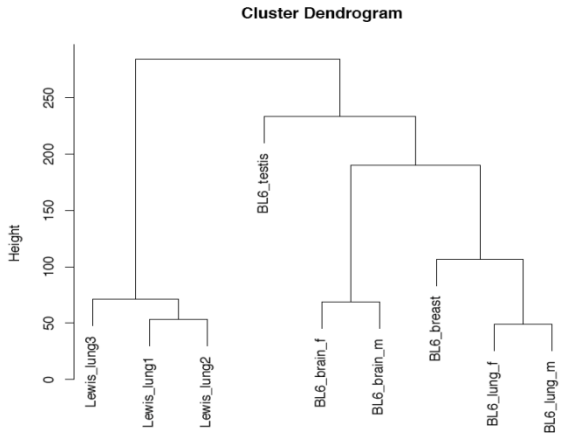

Figure 1. This dendrogram was generated using hierarchical clustering based on a Euclidean distance matrix. The larger the Y-axis value, the greater the biological difference between samples. The different C57BL/6 samples (from left to right), biological replicates of Lewis lung tumors, testis, brain (female and male), breast (female) and normal lung (female and male) were compared against a pool of normal tissues.

We found that most transposon transcripts are in the sense orientation, implying that expression is not exclusively due to read-through, a mechanism by which transposons are expressed due to their internalization into a transcribed gene. If random read-through was prominent then we would see equal distribution of sense and antisense TE transcripts but a prominence of sense-oriented transcripts argues against it. We observed the surprising finding that different normal mouse tissues displayed highly distinct retro-element patterns of expression. This promotes the idea that TE expression can be a good delineating variable for differentiating tissues which we assume can be extrapolated to different complex tumors. These findings with normal tissues suggest non-random somatic expression of these transposons, both independently and as a part of “host” transcripts.

This pilot study demonstrated tight clustering of replicates with relatively minor differences between male and female tissues or between different organs (except for testis as expected based on hypomethylation of germ cells). However, there were striking differences between LLC replicates compared to normal (fig 1). Table 1 lists the top 10 differentially expressed TE transcripts from the three LLC replicates compared to the normal pool, showing a high degree of concordance. With the exception of the L32 pseudogene, all of the hits belong to families that have active retrotransposons. We are currently designing primer sets for qRT-PCR confirmation of differential expression. Although we will focus on data generated from primary cancers, it is encouraging to find such a striking profile in the widely studied LLC model. First described in 1951 by M.R. Lewis and adapted to cell cuture in 1980 by Bertram and Janik, a Pubmed search for “Lewis Lung Carcinoma” yields 3,121 references, but remarkably, when crossed with the terms “endogenous retrovirus” or “transposable elements”, no references are found. Going forward, comparisons will be made

between cancers arising in the KP models and between early atypical adenomatous hyperplasia, grade 2 adenomas, grade 3 adenocarcinomas, grade 4 invasive adenocarcinomas, and frank metastases, with the goal of defining a relatively small set of TEs in which one or more are expressed in >95% of early stage cancers. Initial analysis will seek to evaluate the concordance of TE expression between cancers of the same stage arising in different individual mice, and between 2 or more tumors arising in the same individual.

LEWIS LUNG -Sample 1	
5.435676	ERVB4_3-LTR_MM_ERV2_Mus_musculus
4.897534	ERVB7_1-I_MM_Endogenous_Retrovirus_Mus_musculus
4.736328	MMETN_Endogenous_Retrovirus_Mus_musculus
4.093380	ETNERV_ERV2_Mus_musculus
3.994208	IAPEZI_ERV2_Mus_musculus
3.809462	IAPLTR1a_I_MM_ERV2_Mus_musculus
3.658623	RLTR1IAP_MM_LTR_Retrotransposon_Mus_musculus
3.629766	L32_Pseudogene_Mus_musculus
3.527725	IAPLTR4_I_ERV2_Mus_musculus
3.440774	IAP1-MM_LTR_ERV2_Mus_musculus
LEWIS LUNG- Sample 2	
4.791114	MMETN_Endogenous_Retrovirus_Mus_musculus
4.491925	ERVB7_1-I_MM_Endogenous_Retrovirus_Mus_musculus
4.364071	ETNERV_ERV2_Mus_musculus
3.559637	RLTR10D2_ERV2_Mus_musculus
3.525371	ERVB4_3-LTR_MM_ERV2_Mus_musculus
3.486268	L32_Pseudogene_Mus_musculus
3.429178	RLTR1IAP_MM_LTR_Retrotransposon_Mus_musculus
3.404469	MMERVK10D3_LTR_ERV2_Mus_musculus
3.400826	IAPEZI_ERV2_Mus_musculus
3.380169	RLTR1E_MM_ERV1_Mus_musculus
LEWIS LUNG- Sample 3	
4.891455	ERVB7_1-I_MM_Endogenous_Retrovirus_Mus_musculus
4.661069	MMETN_Endogenous_Retrovirus_Mus_musculus
4.406539	ERVB4_3-LTR_MM_ERV2_Mus_musculus
4.073890	IAPEZI_ERV2_Mus_musculus
4.028246	ETNERV_ERV2_Mus_musculus
3.946389	L32_Pseudogene_Mus_musculus
3.909251	RLTR1IAP_MM_LTR_Retrotransposon_Mus_musculus
3.898549	IAPLTR1a_I_MM_ERV2_Mus_musculus
3.664598	IAPLTR4_I_ERV2_Mus_musculus
3.563673	RLTR2IAP_MM_LTR_Retrotransposon_Mus_musculus

Table 1. The top hits were obtained by ordering the absolute value of the (log2) difference of tumor cell and normal tissue samples against a pool of normal tissues. The ERV's that were implicated in Lewis lung tumor belong to families that have several active and potential functionally retrotransposons

Task 2- *Collection of tumors from K and KP transgenic mouse models.*

A colony of "KP" mice (described in the application) were bred and typed to obtain a

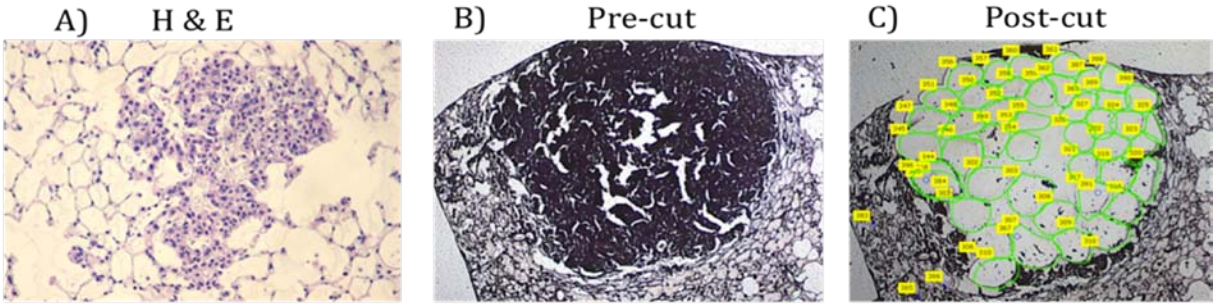

Figure 2. A) Lung section from KP mouse 4 weeks post adenoviral Cre infection. B) 7 micron thick LCMD slides from lung section 16 weeks post adenoviral-Cre infection pre- and C) post-laser cature for RNA isolation.

cohort of 30 mice homozygous for floxed p53 and heterozygous for LSL mutant Kras. At 8 weeks of age, all mice underwent endotracheal instillation of recombinant adenovirus encoding the Cre recombinase. Five mice were sacrificed every two weeks, starting two weeks after adeno-cre administration. Tissue fixation, processing, histopathological classification, laser capture micro-dissection, and RNA isolation was performed in the intervals between sacrifice timepoints. Pre-malignant lesions consistent with adenomatous hyperplasia were observed at 2 weeks post induction in 3 of 5 mice, whereas 100% of mice had well defined lesions by 4 weeks (see figure 2A), including invasive adenocarcinomas

seen as early as 6 weeks post induction. To evaluate the yield and integrety of RNA isolated from FFPE lung tissue blocks, LCMD was performed on samples from week 16 mice, harvesting 36 mm^2 x 7 um thick samples/ mouse, which yielded an average of 1642.92 ng RNA/ sample. RNA was of good quality (average RIN of 4.6, rRNA ratio (28s/18s): 6.8, A260/280 2.01, A260/230 2. 02). This RNA was amplified and aRNA banked for subsequent analysis.

Task 3- *Performing TE gene expression profiling on collected materials*.

Whereas the above pilot demonstrated that the arrays performed well when RNA was abundant, the amount of starting material obtained from the earliest tumor lesions (in which there is the greatest interest) was inadequate to conduct sufficient replicates to assure reliable results using this platform. Accordingly, we have chosen to save the banked materials and are moving to “next generation” whole transcriptome shotgun sequencing (RNA-seq). However, we have continued to use the arrays to “work up” the more abundant (late stage) tumors and LLC, based on our encouraging preliminary data, and in particular, the comparison of normal lung and Lewis lung have implicated Etn and IAPs, two known active endogenous retrovirus (ERVs) transposable elements in mice, both of which were found to be expressed in the KP week 16 tumors.

KEY RESEARCH ACCOMPLISHMENTS:

- Created novel microarray canvassing expression of endogenous retroviral (and other TE) transcripts.
- Demonstrated reproducible detection of differentially expressed TE transcripts in normal organs versus cancers.
- Generated a “rank order” list of candidate TE transcripts as potential cancer neo-antigens.
- Optimized tumor induction, microdissection, and RNA isolation from KP mouse lung cancer model.
- Found concordance of two TE transcripts as being over expressed in both a transplantable mouse lung cancer cell line (LLC) and primary mouse lung cancers (week 16 KP tumors).
- Defined a limitation of the microarray platform precluding its use for examining TE transcript profiling in early stage tumors.

REPORTABLE OUTCOMES:

A report of the array design and performance, with a focus on normal tissue patterns of TE expression is being submitted for publication (see below).

CONCLUSION:

TE expression patterns appear to be reproducible in normal mouse organs, and profoundly differentially expressed in mouse lung cancers. Such transcripts may encode antigens that would be candidates for prophylactic cancer vaccine approaches to cancer prevention.

REFERENCES: TE-Array—A high throughput tool to study transposon transcription Veena P. Gnanakkan, Andrew E. Jaffe, Lixin Dai, Jie Fu, Han Sun, Peilin Shen, Sarah J. Wheelan, Rafael Irizarry, Hyam I. Levitsky, Kathleen H. Burns, Jef D. Boeke*. In preparation.

APPENDICES:

List of personnel (not salaries) receiving pay from the research effort.

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